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2. Patent application number
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   0219143.5 16 AUG 2002

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

   University of Leicester
   University Road
   Leicester
   LE1 7RH

   Patents ADP number *(if you know it)*

   GB

   If the applicant is a corporate body, give the country/state of its incorporation

   7983482001

4. Title of the invention

   Modified Tailed Oligonucleotides

5. Name of your agent *(if you have one)*

   "Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

   McNeight & Lawrence
   Regent House, Heaton Lane
   Stockport, Cheshire SK4 1BS

   Patents ADP number *(if you know it)*

   0001115001

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11. I/We request the grant of a patent on the basis of this application.

Signature: McNeight & Lawrence — Date: 15 August 2002

12. Name and daytime telephone number of person to contact in the United Kingdom: Graeme D McCallum 0161 480 6394

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Modified Tailed oligonucleotides

The present invention relates to modified nucleic acid molecules that are used to provide positively acting RNA processing signals in *trans*.

Antisense methods are widely used to inhibit gene expression in eukaryotic cells. From the therapeutic point of view, one of the most promising developments has been the use of modified and more stable oligonucleotides, for example 2'-*O*-methyl derivatives of RNA, which can be taken up by cells and will anneal to a specific target mRNA to block its expression. In principle, any target gene can be down-regulated by such reagents. A variation of the method has been used to prevent the incorporation of a specific block of RNA into the mature mRNA by preventing splicing of particular exons from the precursor (pre)-mRNA molecule. This may have therapeutic uses in some diseases, such as muscular dystrophy. Indeed, Dunckley *et al.* have shown that a severe dystrophy caused by a mutation that introduced a translational stop codon could be alleviated in principle by the use of antisense oligonucleotides that blocked the splicing of that exon (Dunckley, M. G., Manoharan, M., Villiet, P., Eperon, I. C., and Dickson, G., 1998, Hum. Mol. Genet. 7(7): p1083-1090, PMID: 9618164).

Practically all of the existing methods of modifying the expression of endogenous genes result in a reduction of expression or reduction in the incorporation of particular (deleterious) exons. Short of introducing a correct gene, no general methods are available for enhancing expression or correcting the effects of splicing-related mutations on the basis of knowing only the sequences of the wild-type and (if any) related genes.

Alternative pre-mRNA splicing is a fundamental mechanism for regulating the expression of a multitude of eukaryotic genes. The basic splicing signals, which include the 5' splice site, branch site, and polypyrimidine tract-AG, are initially recognized

by the U1 small nuclear ribonucleoprotein (snRNP), U2 snRNP, U2 snRNP auxiliary factor (U2AF), respectively, and a number of other proteins. These basic splicing signals tend to be degenerate in higher eukaryotes and cannot alone confer the specificity required to achieve accurate splice site selection. Various types of exonic and intronic elements that can modulate the use of nearby splice sites have now been identified. Among the best known examples of such elements are the exonic splicing enhancers - sequences naturally present in pre-mRNA that stimulate the splicing of pre-mRNA transcripts to form mature mRNAs (Cartegni, L., *et al.*, (2002), Nat. Rev. Genet. 3(4): p285-298, PMID: 11967553; Cáceres, J. F. and Kornblihtt, A. R. (2002) Trends Genet. 18(4): p186-193, PMID: 11932019). The definition of "enhancer" is functional, and includes sequences within exons that are not located at the splice sites and are not universally obligatory but do stimulate splicing at least in the gene in which they were identified. Enhancers are commonly thought of as elements in alternatively spliced exons that compensate in part for weak canonical splicing signals. However, it has been shown recently that even constitutive exons can contain several enhancer sequences. The majority of enhancer sequences identified are rich in purines, although recent selection strategies have shown that more diverse classes of sequence are also functional. In a number of cases, it has been shown that these sequences are recognised directly by specific SR (for serine and arginine-rich) proteins. These RNA-binding proteins play a critical role in initiating complex assembly on pre-mRNA, and are essential for constitutive splicing and also affect alternative splicing both *in vivo* and *in vitro*. It is very likely that other proteins, such as Tra2α or β or hnRNP G also play a role in enhancer sequence recognition and/or processing.

Enhancer sequences have also been identified in introns, however general principles concerning their sequence or mode of action have yet to emerge.

In all known cases, enhancer sequences act in *cis*, i.e. they are part of the pre-mRNA substrate. Enhancers can act in *cis* within a partial substrate, where a substrate

lacking a 3' exon has undergone the first step of splicing and then a second RNA containing the 3' portion and an enhancer is added. However, there have been no reports of enhancers acting positively in *trans*, and indeed, enhancers are often added in *trans* as competitors to titrate out enhancer binding factors.

Pre-mRNA molecules may also contain cryptic or mutant splice sites, especially 5' splice sites. The 5' splice site is defined by a poorly conserved short sequence around a highly conserved GU (guanine-uracil) dinucleotide. In most cases, there are many similar sequences in the adjacent intron and exon, but the correct site is chosen as a result of a combination of influences: the extent to which the sequences fit the consensus, the positions of exon elements and other splice sites, and the concentration of the various factors that affect 5' splice sites. Numerous genetic diseases result from mutations at the 5' splice site, the consequences of which are either skipping of the exon or the use of some of the other candidate sites (cryptic splice sites). Enhancer defects are difficult to assign and have only recently entered the broader consciousness as possible explanations for the effects of mutations. Well known examples of genetic diseases that arise from mutations affecting splicing include thalassaemias (e.g. OMIM #141900 for haemoglobin-beta locus), muscular dystrophies (e.g. OMIM #310200), collagen defects (van Leusden, M. R. *et al.* (2001), Lab Invest. 81(6): p887-894, PMID: 11406649), and proximal spinal muscular atrophy (SMA) (Monani, U. R., *et al.* (1999), Hum. Mol. Genet. 8: p1177-1183, PMID: 10369862; Lorson, C. L., *et al.* (1999), Proc. Natl. Acad. Sci. USA 96: p6307-6311, PMID: 10339583).

SMA is an autosomal recessive disorder characterised by muscular weakness and atrophy due to the degeneration of spinal cord motor neurons resulting from mutations of the Survival Motor Neuron (SMN) gene. The SMN gene consists of eight exons, the first seven of which encode a 294 amino acid protein with a molecular weight of 32kDa. The SMN protein is ubiquitously expressed and localised in the cytoplasm and nucleus where it is involved in the process of pre-mRNA splicing. In particular it has a role in the

recycling of snRNPs in the nucleus and probably also in spliceosomal snRNP assembly in the cytoplasm. The SMN gene exists in two copies, a telomeric (SMN1) and a centromeric copy (SMN2). Mutations in SMN1 cause SMA, while the copy number of the residual SMN2 genes is believed to modify the severity of the phenotype. In support of this hypothesis, it has been shown that an increased copy number is associated with a milder disease course. Deletions of both SMN1 and SMN2 have never been observed in humans and a knockout of the single SMN gene in the mouse results in a non-viable embryo. The two genes are 99% identical and differ only by 8 nucleotides, only 2 of which are contained in exons and neither of which alters the coding sequence. The SMN1 and SMN2 genes undergo alternative splicing involving exon 7 and to a lesser extent exon 5, resulting in the SMN1 gene producing primarily full-length SMN transcript whereas the predominant transcript derived from SMN2 lacks exon 7. One of these nucleotide changes is C6T - a T for C substitution at position + 6 in exon 7 of SMN2. This nucleotide is essential for the retention of exon 7 in the mature transcript of the SMN1 gene. This is accomplished by the presence of a high affinity binding site in the SMN1 gene for the SR protein SF2/ASF which generally promotes the inclusion of exons to which it binds (Hastings, M. L. and Krainer, A. R., (2001), Curr. Opin. Cell Biol. 13(3): p302-309, PMID: 11343900). The C6T change found in SMN2 abolishes the ability of this region to bind SF2/ASF, thereby reducing the recognition of exon 7 by the spliceosome, resulting in exon 7 deleted SMN2 transcripts (Cartegni, L. and Krainer, A. R., (2002), Nature Genetics, 4: p377-384, PMID: 11925564).

The retention of intact copies of SMN2 in all SMA patients has led various investigators to devise different strategies for altering the splicing pattern of the SMN2 gene to that of the SMN1 gene, as this might have therapeutic implications for SMA patients. This has been attempted by using pharmacological agents such as sodium butyrate and aclarubicin (Chang, J. G. *et al.*, (2001), Proc. Natl. Acad. Sci. USA; 17: p9808-9813, PMID: 11504946; Andreassi, C., *et al.*, (2001), Hum. Mol. Genet., 24: p2841-2849, PMID: 11734549) or antisense strategies, with oligonucleotides targeted against exon 8 splice

sites, thereby blocking the sites and inducing exon 7 inclusion to a greater extent (Lim, S. R., and Hertel, K. J. (2001), J. Biol. Chem.; 276(48): p45476-45483). However, only a very moderate increase in exon 7 inclusion was achieved by this antisense approach and the drugs involved have potential toxicity problems. Furthermore, the use of antisense oligonucleotides to block an adjacent exon is applicable only in rare cases where this is the 3' terminal exon - if it were an internal exon, the antisense oligonucleotide might lead to skipping of the blocked exon.

The present invention aims to overcome at least one of the prior art disadvantages and contributes significantly to the field by providing a novel product and method for overcoming genetic or induced mutations in RNA molecules that prevent the recruitment of endogenous processing factors to the RNA molecules. An oligonucleotide molecule that comprises an RNA binding domain and an RNA processing factor binding domain is introduced into cells carrying the defective RNA species. The oligonucleotide molecule anneals by means of the RNA binding domain to specific RNA sequences at or near the defective site, and then by means of the RNA processing factor binding domain recruits endogenous RNA processing factors which interact with said RNA species, thereby overcoming the effect of the mutation. This method is universally applicable and requires no further characterisation beyond knowledge of the mutation.

Thus, according to a first aspect of the present invention, there is provided a nucleic acid molecule comprising first and second domains, said first domain being capable of forming a first specific binding pair with a target sequence of a target RNA species, said second domain consisting of a sequence which forms a second specific binding pair with at least one RNA processing or translation factor, said target sequence being sufficiently close on said target RNA species to an RNA processing or translation site for processing or translation at said site to be enhanced by the action of said second domain, and said nucleic acid molecule being arranged such that upon formation of a first specific binding pair with said target sequence, said at least one RNA processing or

translation factor interacts with said RNA target species to form a second specific binding pair at said RNA processing or translation site to effect RNA processing or translation at said RNA processing or translation site.

Regarding the proximity of the target sequence to the RNA processing or translation site on the target RNA species, "sufficiently close" may mean between 0 and 100 nucleotides. For example, the target sequence may be 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 nucleotides from the RNA processing or translation site.

The nucleic acid molecule may be isolated and/or purified.

A "Member of a Specific Binding Pair" is one of two different molecules, having an area on the surface or in a cavity which specifically binds to the other molecule with a particular spatial and polar organization. The members of the specific binding pair are referred to as ligand and receptor (antiligand), sbp member and sbp partner, sbp members or the like. The members of the first specific binding pair can be nucleic acid duplexes, RNA-RNA, and RNA-DNA. The members of the second specific binding pair can be nucleic acid duplexes, RNA-RNA, RNA-DNA, RNA-protein, and the like.

The nucleic acid molecule according to the present invention may be prepared by any convenient method involving coupling together successive nucleotides, and/or ligating oligo- and/or poly-nucleotides, including *in vitro* processes, as well as by recombinant DNA technology (Sambrook, J., Frisch, E. F. and Maniatis, T., 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York).

The nucleic acid molecule may contain multiple functional domains, for example, it may contain binding sites for at least one SR or SR-related protein, and/or at

least one domain to facilitate coupling of the oligonucleotide to additional compounds to enhance uptake into cells and nuclei, e.g. a penetratin binding domain (Derossi, D. *et al.* (1998) Trends Cell Biol. 8(2): p84-87, PMID: 9695814; Derossi, D. *et al.* (1994), J. Biol. Chem. 269(14): p10444-10450, PMID: 8144628).

The nucleic acid molecule may be introduced into target cells using well-known transformation and transfection techniques and reagents e.g. Lipofect Amine (Life Technologies), or GeneJammer (Stratagene), used according to the manufacturers instructions.

The first domain of the nucleic acid molecule may attach to said specific sequence of said RNA target species by means of complementary base pairing. The second domain of the nucleic acid molecule should not be complementary to the RNA target species, so that it is available for the binding of RNA processing factors.

The nucleic acid molecule can comprise at least one modified nucleotide. For example, at least one nucleotide may be chemically modified to enhance stability. Examples of modified nucleotides include those listed in WIPO standard ST25. At least one nucleotide may for example be a 2'-*O*-methyl derivative of RNA and/or a phosphorothioate or morpholino modification. Such modified nucleotides are more stable and less susceptible to attack by endogenous RNAses and other cellular degradation processes. Alternatively or in addition, at least one nucleotide can be modified to enhance uptake of the nucleic acid molecule by a cell, or at least one nucleotide may be modified for any other purpose relating to the improvement of its activity in biological systems.

RNA processing factors may be any RNA or protein that stimulates splicing activity or translation when recruited to the RNA target species at the RNA processing or translation site. The RNA processing factors may comprise any one of the group of: RNA molecules, RNA structural molecules, RNA stability molecules, splicing factors,

polyadenylation factors, transcription factors, and translation factors. These factors may include cellular proteins, nucleic acids, ribonucleoprotein complexes, and combinations thereof.

RNA splicing factors may comprise any one of the group of proteins that influence the site or efficiency of splicing, such as SR proteins, SR-related proteins (Graveley, B. R., (2000), RNA 6(9): p1197-1211, PMID: 10999598), or hnRNP proteins (Krecic, A. M. and Swanson, M. S., (1999), Curr. Opin. Cell Biol. 11(3): p363-371, PMID: 10395553). The RNA sequence binding motifs associated with these proteins are well characterised and are known to a person skilled in the art. Further splicing enhancer sequences known in the prior art (*supra*) may also be utilised.

The nucleic acid molecule may stimulate incorporation of an exon that is normally excluded in a particular cell or tissue, or it may compensate for genetic damage to natural enhancer sequences in the pre-mRNA. The present invention is unique in that *trans*-acting enhancers may be tethered to the pre-mRNA substrate so that the enhancers act positively. For example, in cases of SMA the nucleic acid molecule may stimulate the inclusion of exon 7 in SMN2 transcripts through the recruitment of SR proteins (see Example 1).

The target sequence of a target RNA species may be located within an exon or intron of the target RNA species. It is envisaged that, when there is genetic damage to a 5′ splice site within the 3'-most terminal nucleotides of an exon (for example the three 3'-most nucleotides) or the 5'-most terminal nucleotides of an intron (for example the eight 5'-most nucleotides), the RNA processing factors to be recruited may comprise the U1 snRNP RNA splicing factor, which plays an important role in the recognition of a 5' splice site and the definition of an exon. In the case of mutation within an RNA cryptic splicing site, the RNA processing factors to be recruited may also comprise the U1 snRNP RNA splicing factor. Many splice site mutations are contained within a few nucleotides

preceding the splice site; these are recognised primarily by U1 snRNP and tethering a good U1 binding site nearby may permit use of the correct site.

In addition, the present method may be utilised to stimulate use of the correct splice site in cases where the mutated nucleotide is not recognised by other factors.

It may be advantageous where endogenous mutant and non-mutant isogenes are present to enhance the splicing of the non-mutant form of the gene, altering the ratio of two encoded isoforms. For instance, the gene may be *Ich-1* (encoding Caspase 2), for which the exclusion or inclusion of exon 9 promotes or blocks apoptosis, respectively (Wang, S., *et al.*, (1998), Cell, 92(4): p501-509, PMID: 9491891). Whereas prior art methods for blocking splicing may reduce inclusion, but could not stimulate it, the present method may be used to promote exon inclusion.

Besides modulating splicing, the present invention may also be useful in effecting translation. In eukaryotes, the initiation of mRNA translation is generally thought to occur by a cap-binding/-scanning mechanism. However, some mRNA molecules are translated efficiently in the absence of a free 5' end or cap structure, and some of these mRNA molecules contain sequences within their 5' untranslated regions (5' UTRs) which can directly recruit the translation machinery. Such internal ribosome entry site (IRES) elements have been found in both cellular and viral mRNA molecules. The present invention may be utilised to stimulate translation of a particular transcript by recruiting components of the ribosome or eukaryotic initiation factors, by using recently discovered short IRES sequences (or modules) which can stimulate translation (Chappell, S. A. *et al.*, (2000), Proc. Nat. Acad. Sci. USA, 97(4): p1536-1541, PMID: 10677496). These short 9nt IRES modules are complementary to 18S rRNA sequences (nt 1132-1124) and stimulate translation either alone or synergistically as linked copies by recruiting the 40S ribosomal subunit as a first step in translation of an mRNA (Chappell, S. A. *et al.*, *Supra*). Since virtually all eukaryotic mRNAs are monocistronic and capped, the ability

to internally initiate translation might reflect the ability of a sequence to recruit ribosomes. If the recruitment of ribosomes is sufficient to enhance translation, IRES elements or modules contained within an mRNA may give that mRNA an advantage over other mRNAs which rely on a cap-dependent mode of translation initiation and scanning. Using a modified oligonucleotide wherein the first domain is complementary to a region proximal to the start codon of the transcript, and the second domain contains an IRES module or RNA sequences known to bind initiation factors (such as eIF4G and eIF3) or ribosomal components, it may be possible to promote or stimulate the translation of that particular mRNA. The stimulation of translation of specific transcripts can be of therapeutic benefit in certain disease states, for example, a stimulation of translation of a utrophin transgene can rescue dystrophin deficiency in mice (Rafael, J. A., *et al.*, (1998), Nat. Genet. 19(1): p79-82, PMID: 9590295).

The present invention may also be useful in modulating polyadenylation and thus could offer potential therapeutic benefit to patients infected with retroviruses such as HIV. One of the major strategies required for successful expression of the retrovirus genome is regulation of polyadenylation (poly (A)) signals contained within the long terminal repeats (LTRs) - sequences which flank the viral genome and contain the necessary signals for DNA integration. In the case of HIV-1, both the 5' and 3' LTRs contain poly (A) signals and the virus has evolved ways of selectively activating the poly (A) signal in the 3' LTR, whilst suppressing use of the poly (A) signal in the 5' LTR . This occlusion of poly (A) signal usage in the 5' LTR is achieved through the binding of U1 snRNP to a splice site close to the poly (A) signal (Ashe, M. P., *et al.*, (2000), RNA, 6: p170-177, PMID: 10688356). To reverse this occlusion of polyadenylation by HIV-1, oligonucleotides consisting of a sequence complementary to the HIV-1 RNA sequence close to the poly (A) signal, and a tail sequence or sequences containing motifs designed to recruit polyadenylation reaction components may be used. For example, AAUAAA sequences may be used to recruit cleavage and polyadenylation specificity factor (CPSF), which interacts with cleavage stimulatory factor (CStF), cleavage factor I (CFI), cleavage

factor II and finally poly(A) polymerase (PAP) before cleavage occurs. Since cleavage and polyadenylation are linked, the free 3' ends generated by cleaving are then rapidly polyadenylated. This stimulation of polyadenylation in the 5' LTR could potentially decrease the expression of the HIV-1 genome.

According to a second aspect of the present invention, there is provided the use of a nucleic acid molecule according to the present invention in the manufacture of a medicament for the treatment of RNA processing or translation defects of the human or animal body caused by mutations in RNA that affect binding of RNA processing or translation factors.

According to a third aspect of the present invention there is provided a method for the manufacture of a medicament for the treatment of RNA processing or translation defects caused by mutations in RNA that affect binding of RNA processing or translation factors, characterised in the use of a nucleic acid molecule according to the present invention.

According to a fourth aspect of the present invention there is provided a method for the treatment of RNA processing or translation defects caused by mutations in RNA that affect binding of RNA processing or translation factors, comprising administering to a patient a medicament according to the present invention. Medicaments can include pharmaceutically acceptable carriers, diluents or excipients (Remington's Pharmaceutical Sciences and US Pharmacopoeia, 1984, Mack Publishing Company, Easton, PA, USA; United States Pharmacopoeia, ISBN: 1889788031). The appropriate dosage will be readily apparent to one skilled in the art (based on e.g. dose-response results). The medicament according to the present invention can be administered to a patient in need of same.

Also provided is a method of effecting RNA processing or translation in an *in vitro* system characterised in the use of a nucleic acid molecule according to the present invention. *In vitro* systems can include cell free extracts (see example 1) or cells grown in tissue culture. A nucleic acid molecule according to the present invention can be introduced to such a cell free system and RNA processing allowed to take place such that the nucleic acid molecule effects RNA processing.

The contents of each of the references discussed herein, including the references cited therein, are herein incorporated by reference in their entirety.

Where "PMID:" reference numbers are given for publications, these are the PubMed identification numbers allocated to them by the US National Library of Medicine, from which full bibliographic information and abstract for each publication is available at www.ncbi.nlm.nih.gov.

Where "OMIM:" reference numbers are listed, they refer to the "Online Mendelian Inheritance in Man" database, which is a catalog of human genes and genetic disorders authored and edited by Dr. Victor A. McKusick and his colleagues at Johns Hopkins and elsewhere, and developed for the World Wide Web by NCBI, the National Center for Biotechnology Information. The database contains textual information, references, links to MEDLINE and sequence records in the Entrez system, and links to additional related resources at NCBI and elsewhere (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM).

The invention will be further apparent from the following figures which show, by way of example only, embodiments of the present invention for providing positively acting RNA signals *in trans*.

Of the figures:

Figures 1 and 2 show model representations of the recruitment of RNA splicing enhancer factors according to the present invention;

Figure 3 shows results of an *in vitro* splicing assay incorporating SMN1 and SMN2 transcripts showing alternative splicing. (A) Cell-free *in vitro* splicing assay using [α-^{32}P]-labelled SMN1 transcripts (A, lanes 1-5) and SMN2 transcripts (B, lanes 6-10). Transcripts were incubated at 30°C for 0, 30 minutes, 1, 2 or 3 hours, before termination of the reactions. The reactions were then ethanol precipitated and mixed with 5 µl F-dyes and 3 µl was loaded and fractionated on a 5% denaturing polyacrylamide gel. Lanes 1-5 and 6-10 represent different time points: lanes 1, 6: 0 minutes; lanes 2, 7: 30 minutes; lanes 3, 8: 1 hour; lanes 4, 9: 2 hours and lanes 5, 10: 3 hours. (B) Timed assay of SMN1 transcripts (A, lanes 1-5) and SMN2 transcripts (B, lanes 6-10) using a transcript containing a longer exon 3. This allows the band corresponding to exon 2 spliced to exon 3 to be separated from the splice intermediate of exon 2 spliced to exon 7. In the previous figure these two bands run together on the gel. Lanes 1-5 and 6-10 represent different time points: lanes 1, 6: 0 minutes; lanes 2, 7: 30 minutes; lanes 3, 8: 1 hour; lanes 4, 9: 2 hours and lanes 5, 10: 3 hours. This figure also shows the three different splicing pathways that occur (C, D, and E) - pathways C and D promote exon 7 inclusion while pathway E skips exon 7;

Figure 4 shows a diagrammatical representation (not to scale) of a tailed oligonucleotide bound to SMN2 exon 7 (2). Intron 6 (1), and Intron 7 (3) are also shown. The complementary RNA sequence of the oligonucleotide (A) is in upper case, while the tail region containing sequences that mimic exonic splicing enhancers are in lower case (B). The oligonucleotide binds via a complementary region (A) to the first part of SMN2 exon 7 (2), the non-complementary tail region (B) remains unbound and is thus available to bind to splicing proteins present in the *in vitro* splicing reaction mix;

Figure 5 shows tailed 5'GAA and 5'GGA oligonucleotides promote exon 7 inclusion. (A) Cell-free *in vitro* splicing assay using [α-^{32}P]-labelled SMN2 transcripts combined with oligonucleotide 5'GAA (lanes 2-6, A), oligonucleotide 5'GGA (lanes 7-11, B) and oligonucleotide NT (no tail region) (lanes 12-16, C). The oligonucleotides were either not included in the splicing reactions (lanes 2, 7, and 12), or incorporated at 50 nM (lanes 3, 8, and 13), 100 nM (lanes 4, 9, and 14), 200 nM (lanes 5, 10, and 15) or 250 nM (lanes 6, 11, and 16), respectively. The splicing reactions were allowed to proceed for 3 hours before termination of the reactions. The reactions were then ethanol precipitated and mixed with 5 µl F-dyes and 3 µl was loaded on a 5% denaturing polyacrylamide gel. The SMN1 transcript was included in lane 1. The lariat produced from intron 2 is shown with an arrow. (B) Graph showing relative proportion of exon 7 inclusion (y-axis) relative to the SMN1 level of splicing with increasing concentrations of oligonucleotides (0-250 nM, x-axis). Data points marked with diamonds correspond to the 5' GAA oligonucleotide, data points marked with squares correspond to the 5' GGA oligonucleotide, and data points marked with triangles correspond to the NT oligonucleotide. The SMN1 transcript was included in all gels as an internal control enabling successive gels to be directly correlated. The results of three experiments were combined to produce this data. Standard deviations varied from 0.03 to 0.86;

Figure 6 shows the application of 5'PTB and 5'A1 oligonucleotides to SMN2 transcripts. (A) Cell-free *in vitro* splicing assay using [α-^{32}P]-labelled SMN2 transcripts combined with oligonucleotide 5'GAA (lanes 2-6, A), oligonucleotide 5'PTB (lanes 7-11, B) and oligonucleotide 5'A1(lanes 12-16, C). The oligonucleotides were either not included in the splicing reactions (lanes 2, 7, and 12), or incorporated at 50 nM (lanes 3, 8, and 13), 100 nM (lanes 4, 9, and 14), 200 nM (lanes 5, 10, and 15) or 250 nM (lanes 6, 11, and 16), respectively. The splicing reactions were allowed to proceed for 3 hours before termination of the reactions. The reactions were then ethanol precipitated and mixed with 5 µl F-dyes and 3 µl was loaded on a 5% denaturing polyacrylamide gel. The SMN1 transcript was included in lane 1. (B-D): Graphs showing the percentage of RNA (y-axis)

in the initial pre-mRNA transcript (data points marked with diamonds), the exon 7 included product (data points marked with squares) and the skipped product (data points marked with triangles) at increasing concentrations (x-axis) of 5'GGA oligonucleotide (Figure 6B), 5'PTB oligonucleotide (Figure 6C), and 5'A1 oligonucleotide (Figure 6D). The products have been corrected for the numbers of labelled radionucleotides in each form of RNA. These graphs were plotted from a single experiment, but the results were reproducible in at least three different experiments;

Figure 7 shows the application of 'tail only' oligonucleotides to the SMN2 transcript in the *in vitro* system. (A) Cell-free *in vitro* splicing assay using [α-^{32}P]-labelled SMN2 transcripts combined with oligonucleotide 5'GAA-TO (lanes 2-6, A), oligonucleotide 5'PTB-TO (lanes 7-11, B) and oligonucleotide 5'A1-TO (lanes 12-16, C). The oligonucleotides were either not included in the splicing reactions (lanes 2, 7, and 12), or incorporated at 50 nM (lanes 3, 8, and 13), 100 nM (lanes 4, 9, and 14), 200 nM (lanes 5, 10, and 15) or 250 nM (lanes 6, 11, and 16), respectively. The splicing reactions were allowed to proceed for 3 hours before termination of the reactions. The reactions were then ethanol precipitated and mixed with 5 µl F-dyes and 3 µl was loaded on a 5% denaturing polyacrylamide gel. The SMN1 transcript was included in lane 1. (B-D) Graphs showing the percentage of RNA (y-axis) in the initial pre-mRNA transcript (data points marked with diamonds), the exon 7 included product (data points marked with squares) and the skipped product (data points marked with triangles) at increasing concentrations (x-axis) of 5'GAA-TO oligonucleotide (Figure 7B), 5'PTB-TO oligonucleotide (Figure 7C), and 5'A1-TO oligonucleotide (Figure 7D). The products have been corrected for the numbers of labelled radionucleotides in each form of RNA; and

Figure 8 shows enrichment of splicing reactions with recombinant Tra2 proteins. (A) 5% polyacrylamide gel showing the effect on SMN splicing of enriching the HeLa cell extract with recombinant Tra2 protein, both in the absence and presence of the antisense oligonucleotides. The proteins were added at a final concentration of 1 pM and

preincubated for 10 minutes at 30°C with the HeLa cell extract. The first two lanes show the SMN1 and SMN2 transcripts, respectively, without the presence of oligonucleotides or added SR proteins. Lane 3: SMN2 + GAA oligo; lane 4: SMN2 + Tra2; lane 5: SMN2 + GAA+ Tra2; lane 6: SMN2 + GGA oligo; lane 7: SMN2 + GGA+ Tra2; lane 8: SMN1+ Tra2. (B) Bar chart showing the results of enriching the HeLa cell extract with recombinant Tra2. X-axis: relative proportion of exon 7 inclusion, y-axis (bars 1-8) corresponds to lanes 1-8 in Figure 8A.

In a general embodiment (100) of the present invention (Figure 1), the splicing of a particular exon (5) of an pre-mRNA transcript (6) is stimulated by attachment of a modified oligonucleotide (1) with exogenous enhancer sequences to the exon (5). Exon (5) is defined at its 5' end by a splice site (10) adjacent to intron (7), and at its 3' end by splice site (9) adjacent to intron (8). The modified oligonucleotide (1) has a first exon-annealing domain (2) and a second domain (3) with a sequence known to act as a splicing enhancer. After entry into the target cell, the first domain (1) anneals to the complementary sequence of the exon (5). The second domain (3) recruits enhancer proteins (4), causing the level of splicing (indicated by arrow 11) at the splicing site (9) to increase. This may be done to alter expression in specific tissues, or to counteract mutations in or around the exon that have led to it being excluded during splicing.

In a second general embodiment (200) of the present invention (Figure 2), a modified oligonucleotide (1) is tethered close to either a cryptic splice site (15) and/or a normal splice site (9) such that the recruiting domain (3) of the oligonucleotide (1) behaves as though it were part of the target pre-mRNA transcript (6) itself. Modified oligonucleotide (1) has a first exon-annealing domain (2), complementary to a sequence on exon (5), and a second domain (3) with a sequence that recruits the splicing protein U1 snRNP (4). Tethering of U1 snRNP (4) to a location near to the cryptic splice site (15) and/or the splice site (9) activates either or both sites, causing an increasing in splicing (indicated by arrow 11).

Experiments

Example 1

The following example relates to spinal muscular atrophy and details the use of a novel strategy to modify the splicing of SMN2 that is, in principle, widely applicable to exons that are included at sub-optimal levels. Oligonucleotides have been designed that, while they are complementary to the target exon, do not block reactions at their binding sites like conventional antisense RNA. Instead, the oligonucleotides incorporate a non-complementary 'tail' consisting of sequences that mimic exonic splicing enhancers. We show here that these tailed oligonucleotides induce the inclusion of SMN2 exon 7 with high efficiency, probably via the recruitment of hTra2-ß1 and other proteins. This novel approach has potential therapeutic implications for SMA, but also more generally for the study and modification of splicing regulation.

Methods

ß-Globin/SMN Constructs for use in Cell-free Splicing Assays

Rabbit ß-Globin exon 2, intron 2 and the beginning of exon 3 was amplified by PCR using the novel primers BGEX2F and BGEX3R (see Table 1 for sequences). This PCR product was cloned into the TOPO cloning vector (Invitrogen). SMN1 and SMN2 exons 7 and flanking regions were PCR amplified from previously sequenced clones. Novel primers SALRIIF and SALSMNR were used to create *Sal* I sites. *Sal* I digested PCR products were then cloned into the similarly digested TOPO/ß-Globin vector created previously. The *Sal* I site was situated within the intronic region between the two ß-globin exons such that SMN1 or SMN2 exon 7 and intronic regions were situated between the two ß-globin exons.

Site-directed Mutagenesis of SMN Constructs

The stop codon at the end of SMN exon 7 was altered in order to allow read-through of the ß-globin/SMN constructs. This was achieved by site-directed

mutagenesis using the sequenced SMN constructs obtained above as templates. The vectors were amplified with reverse complementary primers SMN7XF and SMN7XR containing a base pair deletion and a nucleotide change. The PCR was carried out using Pfu turbo polymerase (Hybaid), with the following cycles: 95 °C for 30 seconds, then 12 cycles of 95 °C for 30 seconds, 55 °C for 1 minute and 68 °C for 8 minutes. After successful amplification, the mixture was transformed and the positive clones sequenced.

In-vitro Transcription Mix

Novel primers with the forward primer (T7BGEX2F) incorporating the T7 promoter sequence, and a reverse primer (BGEX3R), situated in ß-globin exon 3, were used to amplify SMN exon 7 and flanking ß-globin exons from the ß-globin/SMN1 and ß-globin/SMN2 constructs, resulting in an 800 bp product. 100 ng of the PCR products were then combined in an *in vitro* transcription mix and the transcripts labelled with [α-^{32}P]-GTP at 37 °C for 3-4 hours. 10 µl Fdyes were then added and the mixture was fractionated on a 5% polyacrylamide gel at 30 W for approximately 1.5 hours.

Visualisation of Transcription Products

The gel plates were separated and the gel exposed to Biomax X-ray film (Kodak) for 1-5 minutes before developing. The transcript bands were excised from the gel, placed in SDS lysis buffer and incubated at 4°C overnight.

***In vitro* splicing**

The radiolabelled transcripts were ethanol precipitated and resuspended in 20 µl TE containing 0.1% RNase inhibitor (RNasin, Promega). A stock splicing mix was made containing 0.5 µl 100 mM ATP; 4 µl 0.5 M Creatine Phosphate; 4 µl 80 mM $MgCl_2$; 2 µl HEPES buffer, pH 7.5; 0.3 µl RNasin and 17 µl 13% Polyvinyl alcohol. Finally, 40 µl HeLa nuclear extract and 20 µl DKCL/DGlu full buffer (Eperon, I. C., *et al.* (2000), Mol. Cell. Biol. 20: p8303-8318, PMID: 11046128) were added to the stock splicing mix. 0.5 µl of each labelled transcript was then aliquoted into PCR tubes and 4.5 µl splicing mix

was added. A timed assay was then carried out with reactions at 0, 30 minutes, 1, 2 and 3 hours, such that an increase in the spliced products can be seen over time. The splicing reactions were placed at 30 °C and removed to the -80 °C freezers at each relevant time point. 50 µl Proteinase K stop mix was added to the thawed reactions and placed at 37 °C for 10 minutes. The samples were then ethanol precipitated and resuspended in 10 µl F-dyes and 3 µl loaded and fractionated on a 5% denaturing polyacrylamide gel (as described previously). The gel was then fixed and dried in a gel drier and exposed for 3-5 hours to a phosphor screen. ImageQuant software (Biorad) was used to quantify the products in experiments using the SMN/ß-globin transcripts. The levels of radioactivity were not corrected to allow for the different numbers of labelled nucleotides in the RNA products.

Splicing protein binding motif-tailed antisense oligonucleotides

A series of 10 tailed antisense oligonucleotides were designed (see table 2 for sequences). They all contained both 2'-O-methyl and phosphorothioate modifications and were obtained from EuroGentec, France. These oligonucleotides were complementary to the 5' end of exon 7 and in addition contained tails designed to recruit various proteins. Two of them (5' GAA and 3'GAA) contained an identical tail situated on either the 5' or 3' end of the oligonucleotide, designed to initially establish the most effective position for the tail. The 5'GAA oligonucleotide was designed to bind to hTra2-ß1, while the 5'GGA oligonucleotide was designed to recruit SF2/ASF. Other tailed oligonucleotides (5' PTB and 5' A1) were designed to recruit polypyrimidine tract binding protein (PTB) and hnRNP A1, respectively. Since these proteins do not stimulate splicing, the 5' PTB and 5' A1 oligonucleotides served as useful negative controls. Other control oligonucleotides contained either no tail (NT), or consisted of a scrambled sequence (Scram). Three oligonucleotides consisting of the tail regions only of 5' GAA, 5' PTB and 5' A1 were also synthesized and used as controls. The oligonucleotides were incorporated to final concentrations of 0, 50, 100, 200 and 250 nM and pre-incubated for 10 minutes with the SMN2 transcript at 30 °C prior to the addition of the splicing mix. The reactions were

allowed to proceed for 3 hours at 30 °C. All experiments were repeated in triplicate and the relative abundance of the spliced products was normalized against SMN1 readings (included as an internal control) and the mean values plotted on a graph.

Enrichment of HeLa cell extract with hTra2ß protein

Recombinant GST-Tra2β was expressed along with the SR protein kinase 1 (SRPK1) in *E. coli* BL21 (DE3). The protein was purified by affinity chromatography using glutathione-agarose beads by incubation in 0.5 M KCl at 30 °C, using standard protocols. GST-tagged Tra2 recombinant protein was preincubated at 30 °C for 10 minutes in the HeLa cell extract splicing mix prior to the addition of the transcripts. The protein had a final concentration of 1 pM and was added to reactions either with or without the GGA or GAA oligonucleotides (used at 250 nM final concentration).

Results

SMN1 and SMN2 transcripts replicate the alternative splicing of endogenous transcripts within an *in vitro* system.

In vitro splicing assays of pre-mRNA containing SMN1 or SMN2 exon 7 and flanking intronic regions can be seen in figure 3A, where the SMN sequences were set between exons 2 and 3 of rabbit ß-globin. Splicing reactions with three exons are relatively difficult to interpret because there are three possible pathways for splicing: skipping, inclusion via splicing of intron 1 before intron 2, and inclusion via splicing of intron 2 before intron 1. To assist in assigning the bands, the splicing experiments were repeated with a longer 3'-most exon (Fig. 3B). Most of the bands could be assigned by direct side-by-side comparisons of the two reactions, with the exception of the faint bands containing a single intron in a lariat. Based on these assignments, we were able to identify the mRNA derived by skipping and inclusion of exon 7. The faint band below the skipped mRNA i.e. ß-Globin exons 2 and 3 spliced together, in Figure 3B is an intermediate in the inclusion pathway that proceeds via splicing of intron 1 before intron 2. With the shorter transcripts, this product co-migrates with the skipped product, but its level is very much

less (Fig. 3B). Thus, we used the shorter transcript for most experiments because it spliced with greater efficiency and ignored the minimal contribution from the intermediate.

The splicing efficiencies of both the longer and shorter transcripts recapitulated the splicing pattern of endogenous human SMN genes. When comparing the intensities of the bands representing exon 7 inclusion with the total amount of spliced product, for the shorter transcript (Fig. 3A), we found that SMN1 exon 7 inclusion was on average 3.5-fold higher (24.6% ± 6.3 mean inclusion) than that of SMN2 (7% ± 0.74 mean inclusion). These figures have not been corrected for the numbers of labelled radionucleotides in each form of RNA, allowing the values to be correlated directly with inspection of the bands seen on the images. The readings varied between 19.0-32.3% for the SMN1 and 6.5-8.1% for the SMN2 transcripts. Exon inclusion appeared to result from both possible pathways, but the majority of transcripts seemed to follow the route in which intron 1 was removed first.

Modified tailed antisense oligonucleotides increase exon 7 inclusion within the SMN2 transcript.

Antisense oligonucleotides were designed that were complementary to SMN exon 7 and contained additional non-complementary sequences (tails) that were predicted to recruit splicing enhancer factors (see Fig. 4). Two of these contained identical tails of GAA repeats on either the 5' or the 3' side of the oligonucleotide. These oligonucleotides were designed to initially establish the most effective position for the tail. The choice of the GAA sequence was based on the known ability of hTra2ß to bind to GAA sequences as well as published experimental evidence that Tra2ß protein is able to bind to the SMN exon and, when transfected into cells, to enhance its inclusion. Another oligonucleotide contained a 5' GGA tail, GGAGGA being a subset of the sequences shown by functional SELEX to mediate the effects of the SR protein, SF2/ASF. Furthermore, (GGA) repeats are a feature of a number of enhancers, including human tropomyosin TPM3. Control oligonucleotides contained either no tail, or consisted of a scrambled sequence. The

oligonucleotides were incubated with the pre-mRNA substrate and then mixed with a splicing reaction mixture. Both the 5'GAA and 5'GGA oligonucleotides reduced the level of the SMN2 exon 7 skipped product even at the lowest concentration (50 nM), and they increased the level of the mRNA product including exon 7 (Fig. 5A). The increase in inclusion was relatively weak with the GAA oligonucleotide (compare lanes 2, without oligonucleotide, and 3) but robust with the GGA oligonucleotide (lanes 8-11). In contrast, the NT (no tail) oligonucleotide had very little effect. The relative proportion of inclusion in SMN2 rose with both the GAA and GGA oligonucleotides to levels higher than for SMN1 (Fig. 5B), although with the GAA oligonucleotide this was largely caused by the inhibition of skipping. Other experiments (not shown) showed that the 5' GAA tail was more effective than the 3' GAA tail, and therefore all other tailed oligonucleotides carried 5' tails. A comparison of the bands produced by splicing in the presence of the 5'GGA oligonucleotide with the pattern of SMN1 splicing shows that the oligonucleotide produces a relatively high level of bands corresponding to pathway 1 linear intermediates containing the first intron (cf. Fig. 3B). This suggests that pathway 1 is promoted, i.e., that removal of intron 2 is accelerated and that the pathway intermediates accumulate because splicing of intron 1 is limiting. Another relatively abundant band can be seen underneath the inclusion mRNA in Figure 5A, which may represent intron 2, but this has not been formally verified.

The increase in SMN2 exon 7 inclusion at low concentrations of oligonucleotide requires an enhancer-like sequence and base-pairing to exon 7.

To test whether the effects seen were a specific consequence of attaching the enhancer-like sequence to SMN2 exon 7, other tailed oligonucleotides were designed that were predicted to recruit inhibitors of splicing such as PTB or hnRNP A1. In contrast to the 5'GGA oligonucleotide, neither 5'PTB or 5'A1 oligonucleotides caused a significant increase in the level of exon 7 inclusion at the lower concentrations (Fig. 6A and B-D). However, whereas the 5'PTB oligo had no effect at any concentration (Fig. 6C), the higher concentrations of 5'A1 produced a marked decrease in the level of skipped mRNA and a

very slight increase in inclusion (Fig. 6D). This is likely to be the result of sequestration of hnRNP A1 by un-annealed oligonucleotide.

To test this, and to establish whether the enhancement of exon 7 inclusion by the 5'GAA oligonucleotide resulted from binding to the pre-mRNA, oligonucleotides consisting of the tail regions only of 5'GAA, 5'PTB and 5'A1 were synthesized. The 'tail-only' oligonucleotides were incorporated in the SMN2 splicing reactions as previously described (Fig. 7A and B-D). The results showed that the 5' A1-TO produced a concentration-dependent decrease in skipping akin to that of the 5'A1 oligonucleotide (Fig. 7D), consistent with the hypothesis that the A1 oligonucleotide was sequestering 'free' hnRNP A1 proteins irrespective of its action on SMN2 exon 7. The 5'GAA-TO oligonucleotide produced a decrease in skipping but it had no effect on exon 7 inclusion (Fig. 7B).

Effects of recombinant Tra2 protein on SMN1 but not SMN2

Recombinant GST-Tra2 protein was added to splicing reactions to determine whether the attachment of the tailed oligonucleotides would increase responsiveness to Tra2ß. The results (Fig. 8A and B) showed that GST-Tra2ß stimulated splicing of SMN1, but not SMN2, even though the putative binding site for Tra2ß is present in SMN2 exon 7 and it responds *in vivo*. The oligonucleotides stimulated exon 7 inclusion as usual, such that the level of inclusion at least matched that of SMN1, but the protein supplement had little effect. We conclude that Tra2ß binding does limit the efficiency of splicing of SMN1, but that the level of binding of SF2/ASF is also likely to be low for SMN2. The oligonucleotides may bind Tra2ß and other activating proteins efficiently, even in the absence of supplements but the level of SF2/ASF binding at the site of the C-T transition remains an additional barrier.

The present inventors have devised a novel strategy that takes advantage of an antisense oligonucleotide approach but, in contrast to the normal use of such

oligonucleotides as physical obstructions of a reaction at a target site or as mediators of RNase H degradation, we have used these oligonucleotides to attach potent enhancer sequences to the SMN exon, which then activate it. The oligonucleotides use exon 7 as a docking site and the unbound tail region sequesters SR proteins to the immediate vicinity of SMN2 exon 7. As SR proteins function in a concentration dependent manner, by increasing the local concentration of SR proteins surrounding exon 7, its inclusion in the final transcript should be increased.

In our *in vitro* model we have characterised a series of oligonucleotides with tails aimed at recruiting hTra2ß and SF2/ASF, with PTB and hnRNP A1 proteins as controls. We show a specific increase in the proportion of exon 7 inclusion in the SMN2 mRNA when using the 5'GGA (aimed at recruiting SF2/ASF) and 5'GAA (hTra2ß) oligonucleotides (Fig. 5A). However, in the case of the 5'GAA oligonucleotide, the effect was largely accounted for by a decrease in the level of skipped mRNA. In both cases, the proportion of exon 7 inclusion was increased by concentrations as low as 40 nM, and it reached a plateau at concentration exceeding 250 nM. A closer examination showed that the level of exon 7 inclusion was almost maximal at the lowest concentrations of oligonucleotide tested and that the higher concentrations caused a decline in the exon-skipped product. The probable explanation for this is that an excess of the oligonucleotide depletes enhancer-binding factors from the nuclear extract. This reduces the efficiency of all splicing events (including skipping), but the oligonucleotide bound to exon 7 allows that exon to compete effectively, permitting exon 7 splicing to continue with relatively little impediment.

The maximum level of exon 7 incorporation achieved with SMN2 was seen with the 5'GGA oligonucleotide, which reproducibly increased the level to approximately the same as that seen with SMN1 or even higher. It has been shown that the C-T change between SMN1 and SMN2 in exon 7 caused the loss of an SF2/ASF binding site. Indeed, analysis of the 5'GGA oligonucleotide with an "ESE finder program" (Cartegni, L. and

Krainer, A. R., *Supra*) revealed that the oligonucleotide possessed high scores for three overlapping putative SF2/ASF binding motif, which were not identified in the 5' GAA oligonucleotide, confirming the crucial role of the SF2/ASF binding domain in the inclusion of exon 7 (Cartegni, L. and Krainer, A. R., *Supra*).

A separate mechanism may underlie the effect on exon 7 inclusion of the 5' GAA oligonucleotide, which is supposed to recruit hTra2-ß1 protein. Enrichment of HeLa cell extract with recombinant Tra2 protein in combination with 5'GAA and 5'GGA oligonucleotides resulted in a specific increase in SMN2 exon 7 inclusion only when the 5' GAA oligonucleotide was present, indicating that GAA repeat motifs rather than GGA are more effective in Tra2 binding. Enrichment of recombinant Tra2 proteins to both the SMN1 and SMN2 transcripts in the absence of any nucleotides did not result in increase SMN2 exon 7 incorporation; surprisingly, however, Tra2 dramatically increased exon 7 inclusion in the SMN1 spliced products (Fig. 8). This was unexpected because the proposed binding site of hTra2-ß1 is identical in both the SMN1 and SMN2 genes. Various mechanisms could explain these findings; a possibility is a different secondary structure and accessibility of the binding sites of hTra2-ß1. Alternatively it is possible that hTra2-ß1 necessitates efficient SF2/ASF binding (as is the case with SMN1 but not SMN2), in order to promote exon 7 inclusion. It has long been accepted that the alternative splicing occurs as a result of a cumulative effect of many different splicing proteins acting both co-operatively and antagonistically with one another to regulate splicing. Indeed, another SR protein SRp30c, is capable of altering SMN2 splicing, but only through co-operation with HTra2-ß1.

The addition of hTra2-ß to the nuclear extract stimulates inclusion in SMN1, but has relatively little effect on SMN2 (Fig. 8A, compare lanes 1 & 8 with lanes 2 & 4), consistent with a limiting level of SF2/ASF binding. However, it also has little effect on SMN2 in the presence of either the 5'GGA or 5'GAA oligonucleotide. This suggests that there are other factors limiting even further improvements in efficiency. There are a

number of possible explanations, and further work will be needed to resolve them. However, achieving an efficiency matching that of SMN1 is in itself potentially of value. A modest increase in exon 7 incorporation was also observed when using the 5' A1 oligonucleotide, while no effect of the 5'PTB oligonucleotide was noticed (Fig. 6A and B-D). It was suspected that the binding of hnRNP A1 would inhibit exon incorporation, since there are several examples of exonic splicing silencers whose effect is mediated by hnRNP A1. A likely mechanism is that hnRNP A1 and SF2/ASF compete for binding to the pre-mRNA, even though they have different high affinity binding sites, hnRNP A1 binding being nucleated by its high affinity sites and its propagation being limited by SF2/ASF. The absence of a high affinity site for SF2/ASF in SMN2 exon 7, caused by the C-T change at position 6, may mean that the exon is already swathed in hnRNP A1 and the effect of the oligonucleotide may be merely that un-annealed oligonucleotide titrates out some of the hnRNP A1. However, the results indicate that there is very little increase in exon 7 incorporation, and the major effect is a decrease in the level of skipped mRNA. This may indicate that (unsurprisingly) the oligonucleotide also sequesters some splicing activators.

The above results show that the use of tailed oligonucleotides containing SR binding motifs is an effective approach for controlling splicing efficiency. This technique has been applied to spinal muscular atrophy, a common genetic disorder and results show that the 5'GGA and 5'GAA antisense oligonucleotides result in very significant inclusion of exon 7 within SMN2 at levels that are comparable to or higher than those of endogenous SMN1. Gene therapy strategies aimed at correcting the alternative splicing present in the SMN2 gene resulting in increased SMN production thus represent a promising means of providing therapeutic benefit to SMA patients. These results also highlight the importance of accessibility of the SR proteins to their sites of action, as enrichment with SR proteins alone did not achieve a correction of the aberrant splicing. In addition, systemic or generalised administration of SR proteins may have a detrimental effect due to their action on multiple genes, as suggested by the toxicity observed in the experiments to produce

stable transfectants expressing SR proteins (Andreassi, C. et al. 2001, Hum. Mol. Genet. 24: p2841-9).

Approximately 15% of point mutations identified produce splicing abnormalities resulting in increased exon inclusion or exclusion ultimately culminating in genetic disease. Our novel approach of using 'tailed' oligonucleotides to alter splicing thus represents a promising new therapeutic approach not only for SMA, but for a variety of genetic disorders.

Table 1: Primers used for cloning

Primer Name	SEQ ID NO.
BGEX2F	1
BGEX3R	2
SALRIIF	3
SALSMNR	4
SMN7XF	5
SMN7XR	6
T7BGEX2F	7

Table 2: Modified tailed oligonucleotides

Name	SEQ ID NO.
5'GAA	8
3'GAA	9
5'GGA	10
5'PTB	11
5'A1	12
NT (no tail)	13
Scram	14

CLAIMS

1. A nucleic acid molecule comprising first and second domains, said first domain being capable of forming a first specific binding pair with a target sequence of a target RNA species, said second domain consisting of a sequence which forms a second specific binding pair with at least one RNA processing or translation factor, said target sequence being sufficiently close on said target RNA species to an RNA processing or translation site for processing or translation at said site to be enhanced by the action of said second domain, and said nucleic acid molecule being arranged such that upon formation of a first specific binding pair with said target sequence, said at least one RNA processing or translation factor interacts with said RNA target species to form a second specific binding pair at said RNA processing or translation site to effect RNA processing or translation at said RNA processing or translation site.

2. A nucleic acid molecule according to claim 1, wherein said first domain of said nucleic acid molecule attaches to said target sequence of said RNA target species by means of complementary base pairing.

3. A nucleic acid molecule according to either of claims 1 or 2, wherein said nucleic acid molecule comprises at least one modified nucleotide.

4. A nucleic acid molecule according to any of claims 1-3, wherein said at least one modified nucleotide is chemically modified to enhance stability or uptake by a cell.

5. A nucleic acid molecule according to claim 4, wherein said at least one modified nucleotide comprises a 2'-*O*-methyl derivative of RNA and/or a phosphothiorate and/or a morpholino modification.

6. A nucleic acid molecule according to any of claims 1 to 5, wherein said RNA processing or translation factors are selected from the group consisting of: RNA molecules, RNA structural molecules, RNA stability molecules, splicing factors, polyadenylation factors, transcription factors, and translation factors, and combinations thereof.

7. A nucleic acid molecule according to any of claims 1 to 6, wherein said RNA processing or translation factor is any RNA or protein that stimulates splicing activity when recruited to the said RNA target species at said RNA processing site to effect RNA processing at said RNA processing site.

8. A nucleic acid molecule according to any of claims 1 to 7, wherein said RNA processing or translation factor is selected from the group consisting of: SR proteins, SR-related proteins, and hnRNP proteins.

9. A nucleic acid molecule according to any one of claims 1 to 8, wherein said RNA processing site is an RNA splicing site and said RNA processing factors comprise the U1 snRNP group of RNA splicing factors.

10. A nucleic acid molecule according to any one of claims 1 to 9, wherein said RNA processing site is an RNA cryptic splicing site and said RNA processing factors comprise the U1 snRNP group of splicing factors.

11. The use of a nucleic acid molecule according to any one of the preceding claims in the manufacture of a medicament for the treatment of RNA processing or translation defects of the human or animal body caused by mutations in RNA that affect binding of RNA processing or translation factors.

12. A method for the manufacture of a medicament for the treatment of RNA processing or translation defects caused by mutations in RNA that affect binding of RNA

processing or translation factors, characterised in the use of a nucleic acid molecule according to any of claims 1 to 10.

13. A method for the treatment of RNA processing or translation defects caused by mutations in RNA that affect binding of RNA processing or translation factors comprising administering to a patient a medicament according to claim 12.

14. A method of affecting RNA processing or translation in an *in vitro* system characterised in the use of a nucleic acid molecule according to the present invention.

ABSTRACT

MODIFIED TAILED OLIGONUCLEOTIDES

A nucleic acid molecule comprising first and second domains, said first domain being capable of forming a first specific binding pair with a target sequence of a target RNA species, said second domain consisting of a sequence which forms a second specific binding pair with at least one RNA processing or translation factor, said target sequence being sufficiently close on said target RNA species to an RNA processing or translation site for processing or translation at said site to be enhanced by the action of said second domain, and said nucleic acid molecule being arranged such that upon formation of a first specific binding pair with said target sequence, said at least one RNA processing or translation factor interacts with said RNA target species to form a second specific binding pair at said RNA processing or translation site to effect RNA processing or translation at said RNA processing or translation site.

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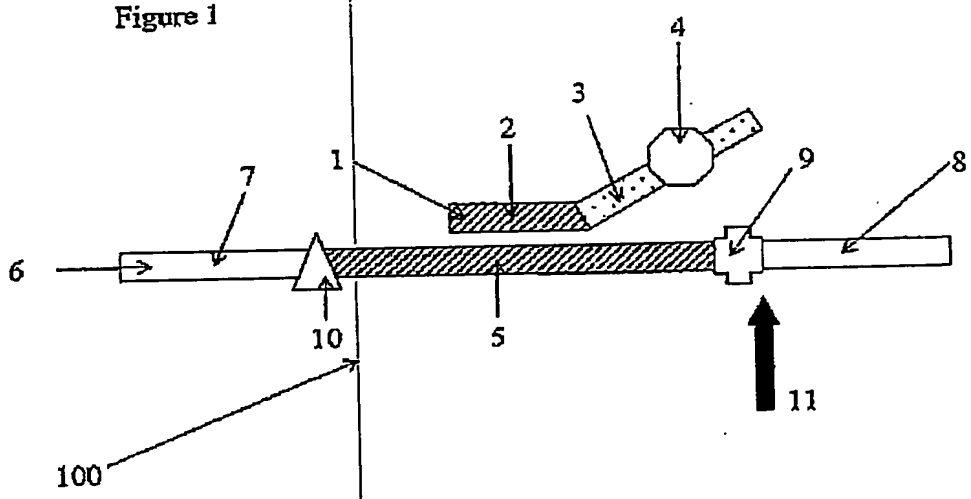

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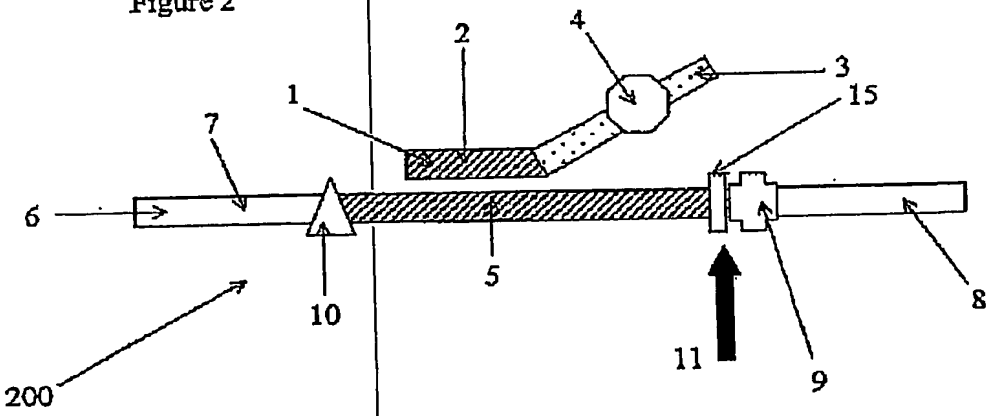

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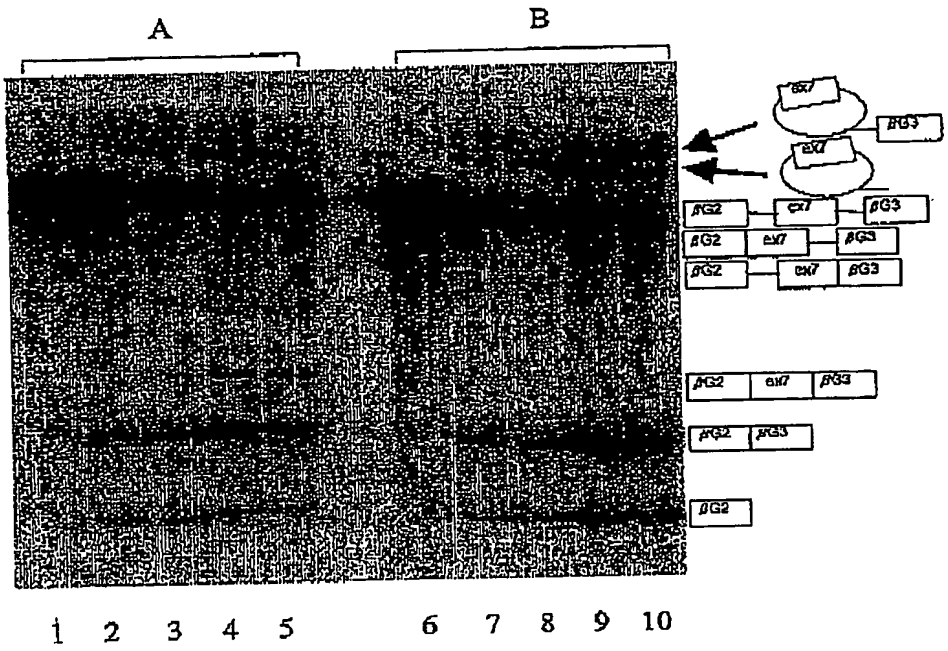

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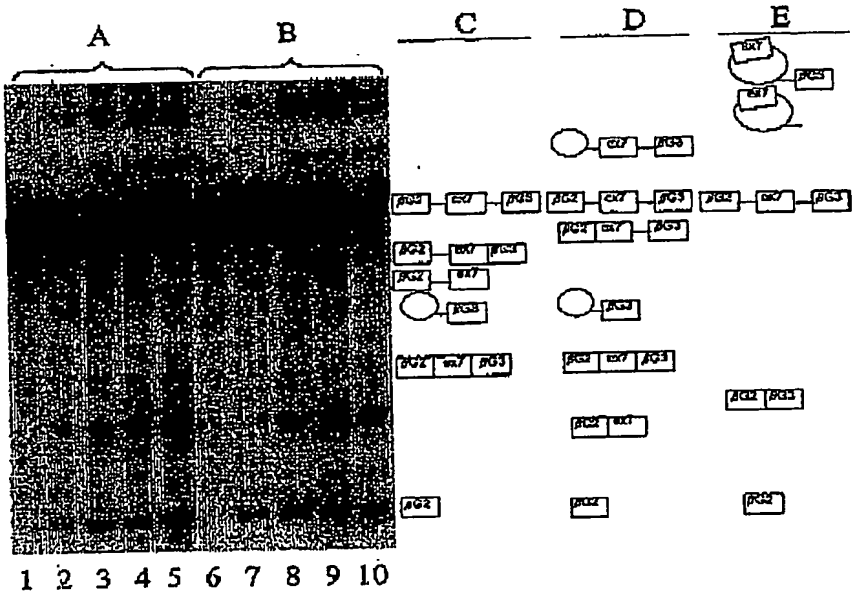

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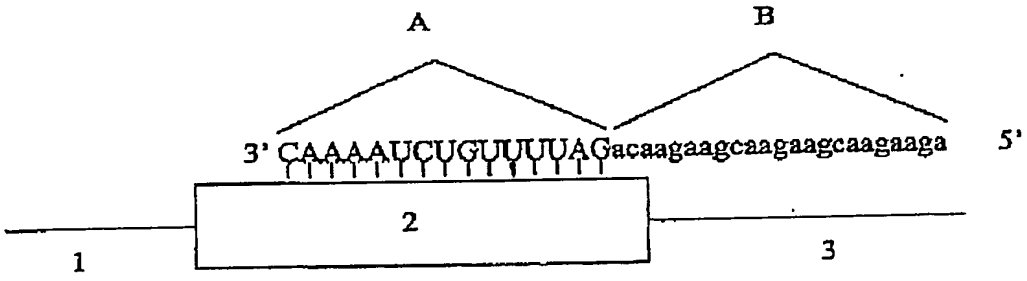

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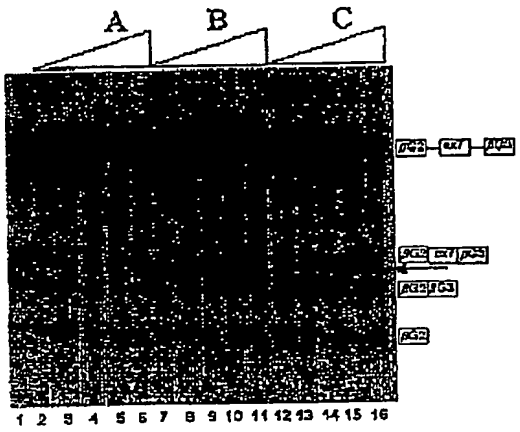

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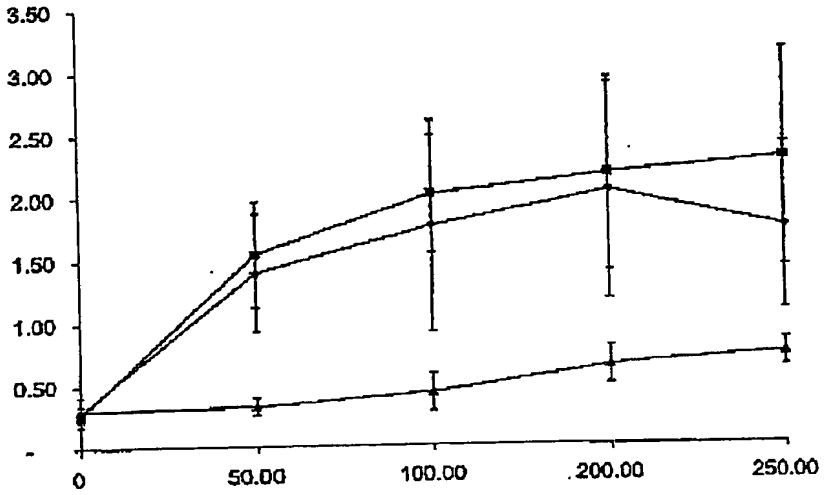

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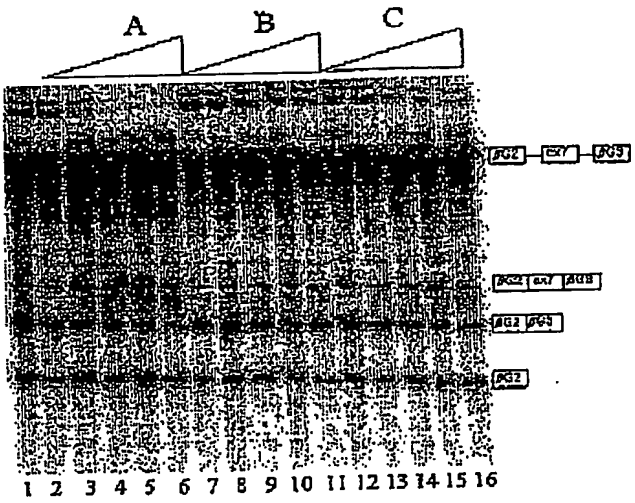

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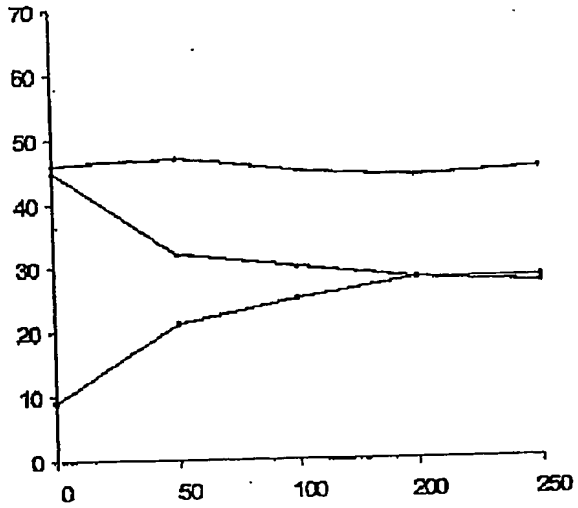

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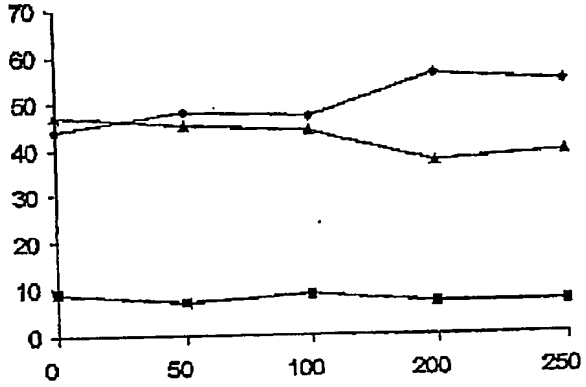

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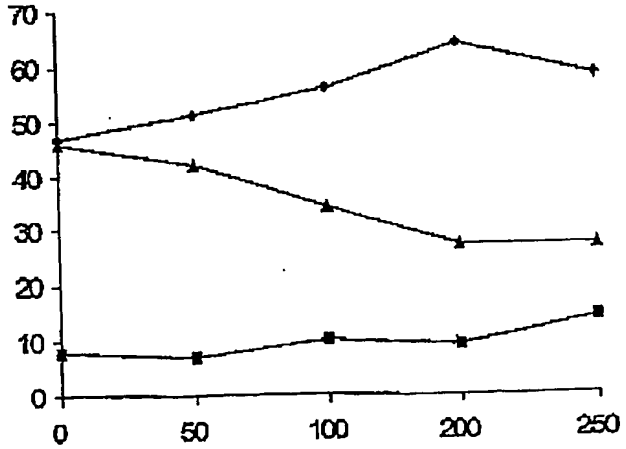

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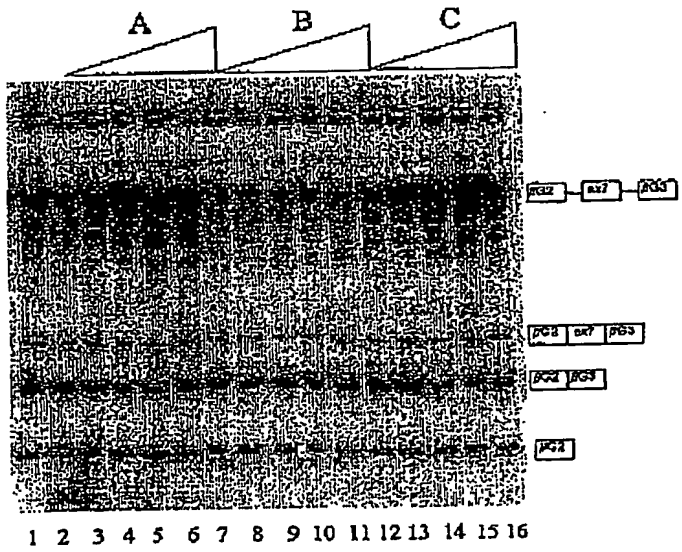

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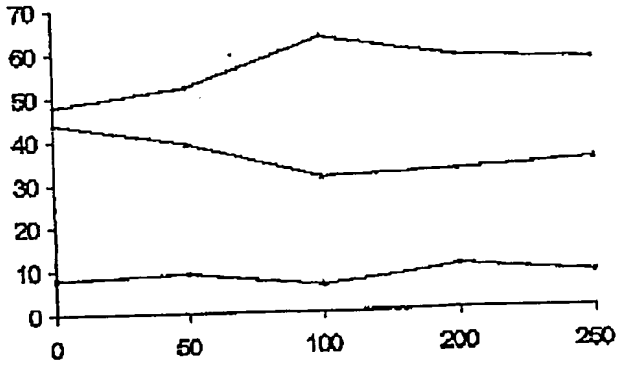

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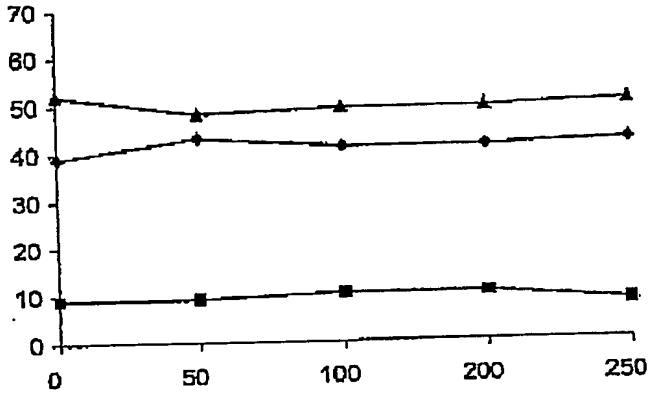

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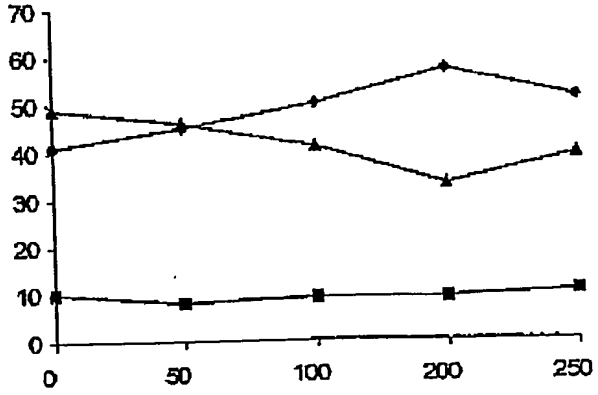

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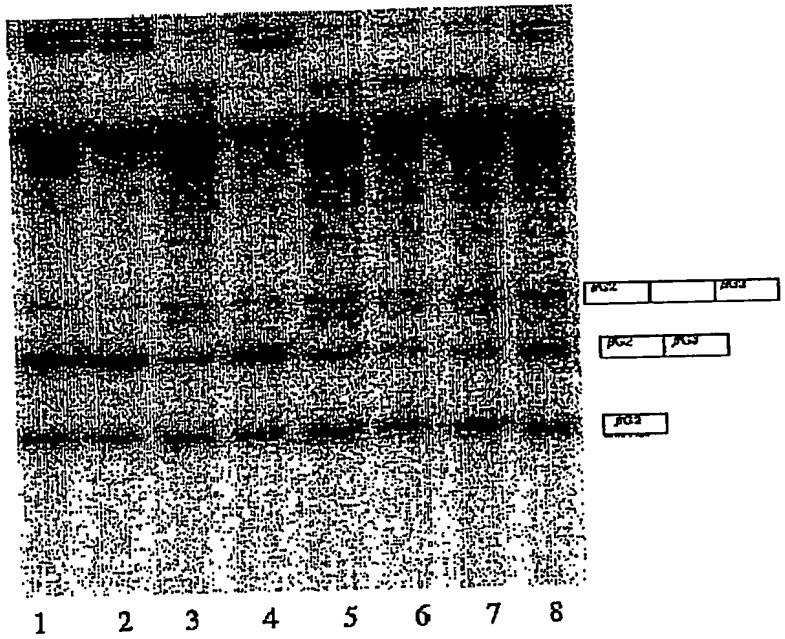

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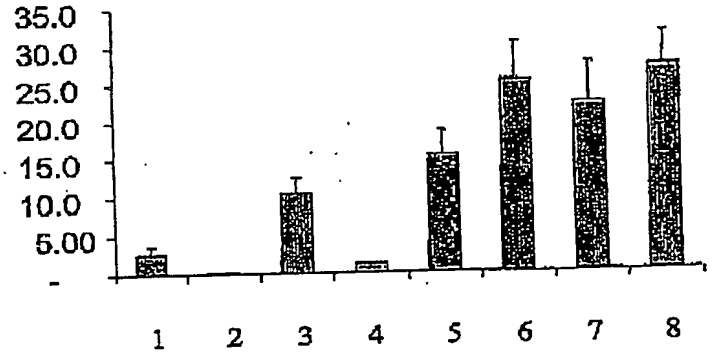

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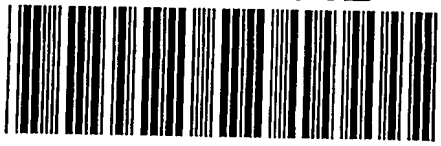

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